Class 13: Transcriptomics and the analysis of RNA-Seq data

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NOTE: we did not follow the questions on the hands-on lab sheet during this session.

Background

Today we will analyze some RNA Sequencing data on the effects of a common steroid drug on airway cell lines.

There are two main inputs we need for this analysis:

- `countData`: counts for genes in rows with experiments in the columns
- `colData`: or metadata that tells us about the design of the experiment (i.e. what is in the columns of `countData`)

Import countData and colData

```
counts <- read.csv("airway_scaledcounts.csv", row.names=1)
metadata <- read.csv("airway_metadata.csv")
```

```
head(counts)
```

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516
ENSG00000000003	723	486	904	445	1170
ENSG00000000005	0	0	0	0	0
ENSG00000000419	467	523	616	371	582
ENSG00000000457	347	258	364	237	318

```
ENSG00000000460          96          81          73          66         118
ENSG00000000938           0           0           1           0           2
                SRR1039517 SRR1039520 SRR1039521
ENSG00000000003       1097         806         604
ENSG00000000005          0           0           0
ENSG00000000419        781         417         509
ENSG00000000457        447         330         324
ENSG00000000460         94         102          74
ENSG00000000938          0           0           0
```

```
head(metadata)
```

```
          id     dex celltype     geo_id
1 SRR1039508 control   N61311 GSM1275862
2 SRR1039509 treated   N61311 GSM1275863
3 SRR1039512 control  N052611 GSM1275866
4 SRR1039513 treated  N052611 GSM1275867
5 SRR1039516 control  N080611 GSM1275870
6 SRR1039517 treated  N080611 GSM1275871
```

Q1. How many genes are in this dataset?

```
nrow(counts)
```

```
[1] 38694
```

Q2. How many 'control' cell lines do we have?

```
table(metadata$dex)
```

```
control treated
      4       4
```

```
sum(metadata$dex == "control")
```

```
[1] 4
```

Toy differential gene expression

Let's try finding the average or mean of the "control" and "treated" columns and see if they differ.

- 1.First we need to find all "control" columns
- 2. Extract just the "control" values for each gene
- 3. Calculate the `mean()` for each gene "control" values

```
all ( colnames(counts) == metadata$id )
```

```
[1] TRUE
```

The $dex column tells me whether we have "control" or "treated"

```
control.inds <- metadata$dex == "control"
```

Extract just the "control" values for all genes

```
control.counts <- counts[,control.inds]
```

Calculate the mean value for each gene in these "control" columns

```
control.mean <- rowMeans(control.counts)
```

Q3. Do the same for "treated" to get `treated.mean`

```
treated.inds <- metadata$dex == "treated"
```

```
treated.counts <- counts[,treated.inds]
```

```
treated.mean <- rowMeans(treated.counts)
```

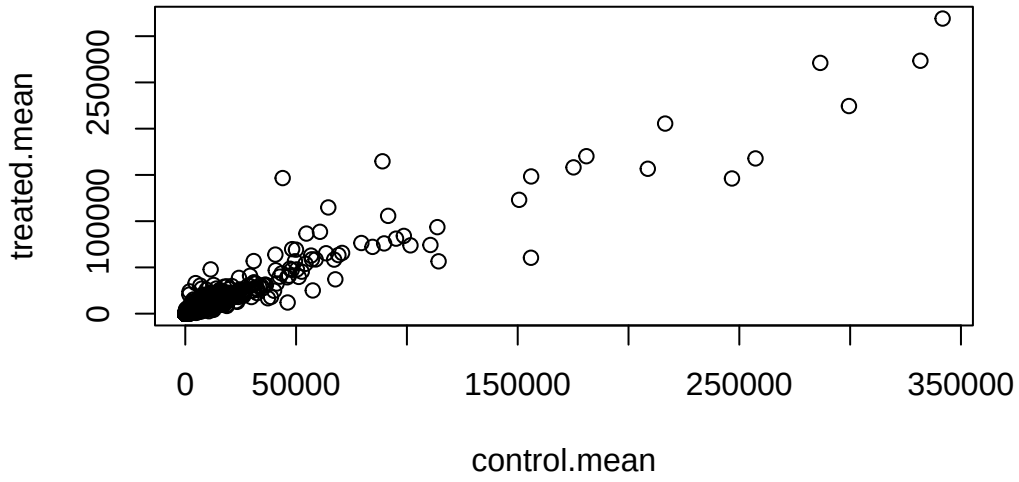
Q4. Make a plot of `control.mean` vs. `treated.mean`

Let's store our mean values together in a data.frame for easier book-keeping

```
meancounts <- data.frame(control.mean, treated.mean)
head(meancounts)
```

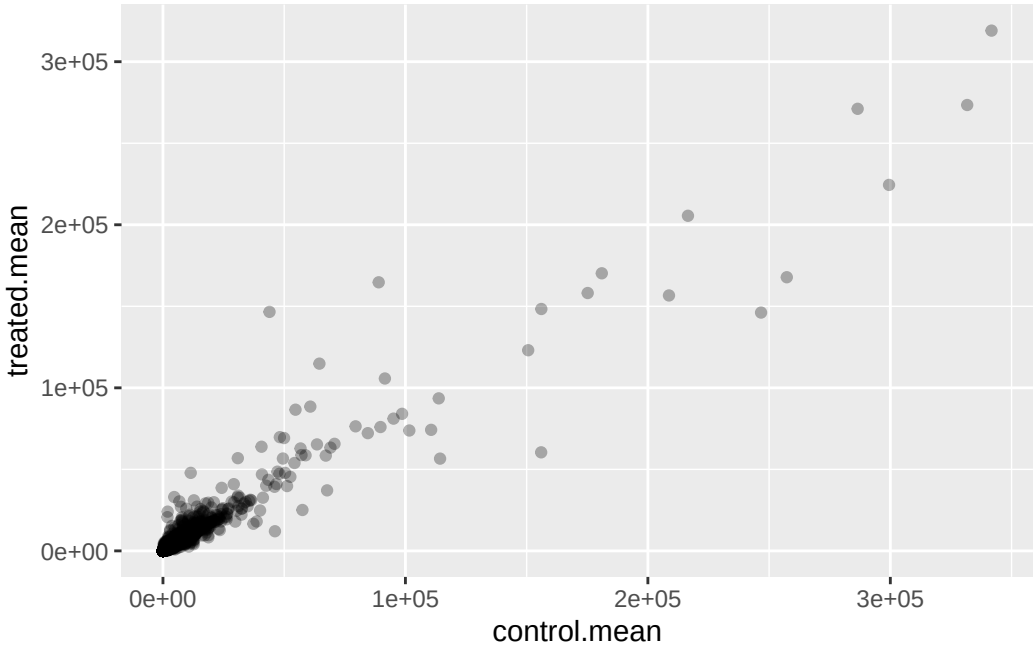
```
                 control.mean treated.mean
ENSG00000000003       900.75       658.00
ENSG00000000005         0.00         0.00
ENSG00000000419       520.50       546.00
ENSG00000000457       339.75       316.50
ENSG00000000460        97.25        78.75
ENSG00000000938         0.75         0.00
```

```
plot(meancounts)
```




```
library(ggplot2)

ggplot(meancounts) +
  aes(control.mean, treated.mean) +
  geom_point(alpha=0.3)
```

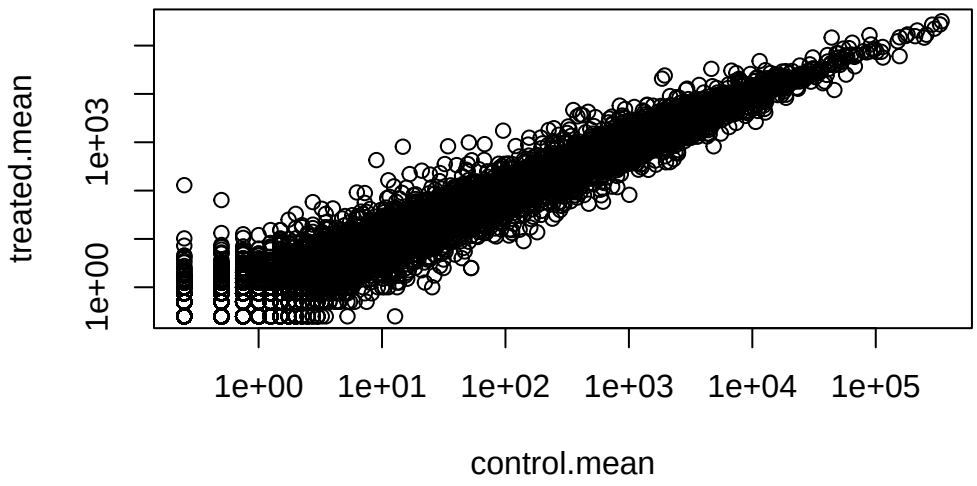


We totally need to log transform this data as it is so heavily skewed!

```
plot(meancounts, log="xy")
```

```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15032 x values <= 0 omitted
from logarithmic plot
```

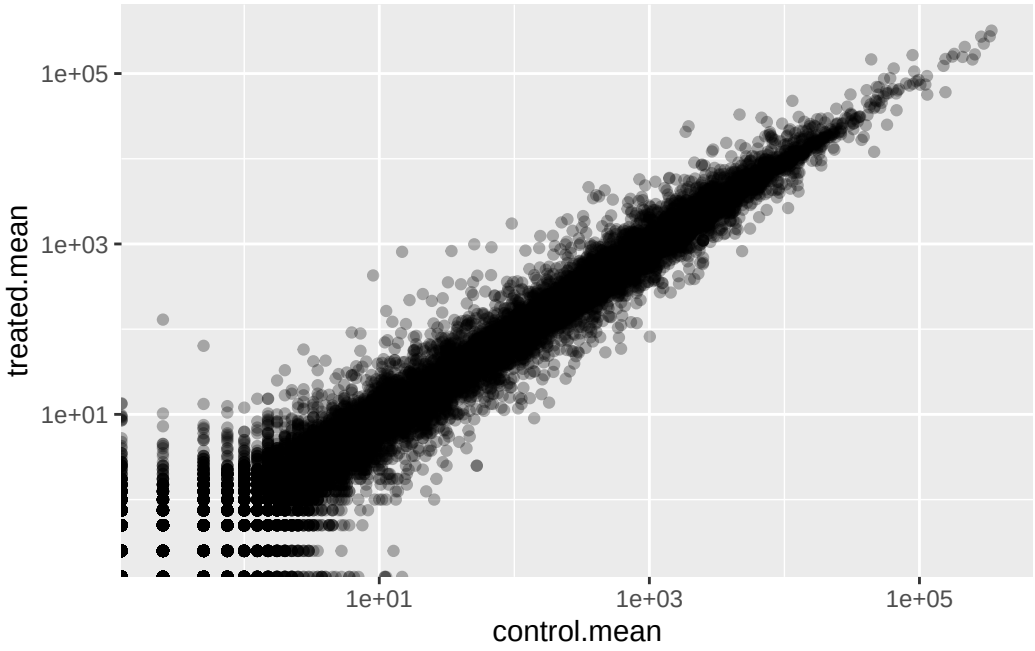
```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15281 y values <= 0 omitted
from logarithmic plot
```




```
ggplot(meancounts) +
  aes(control.mean, treated.mean) +
  geom_point(alpha=0.3) +
  scale_x_log10() +
  scale_y_log10()
```

```
Warning in scale_x_log10(): log-10 transformation introduced infinite values.
```

```
Warning in scale_y_log10(): log-10 transformation introduced infinite values.
```




```
# TREATED/CONTROL

log2(20/20)
```

```
[1] 0
```

Doubling of the amount

```
log2(40/20)
```

```
[1] 1
```

Half of the amount

```
log2(10/20)
```

```
[1] -1
```

A common "rule-of-thumb" is to focus on genes with a log2 "fold-change" of +2 as so-called UPREGULATED and -2 as so-called DOWNREGULATED.

```
log2(80/20)
```

```
[1] 2
```

Let's add a log2 fold-change to value to our **meancounts** data.frame

```
meancounts$log2fc <- log2(meancounts$treated.mean/meancounts$control.mean)

head(meancounts)
```

```
                control.mean treated.mean      log2fc
ENSG00000000003       900.75       658.00 -0.45303916
ENSG00000000005         0.00         0.00         NaN
ENSG00000000419       520.50       546.00  0.06900279
ENSG00000000457       339.75       316.50 -0.10226805
ENSG00000000460        97.25        78.75 -0.30441833
ENSG00000000938         0.75         0.00        -Inf
```

Q5. Remove any "zero count" genes from our dataset for further analysis.

```
to.keep <- rowSums( meancounts[, 1:2] == 0 ) == 0
sum(to.keep)
```

```
[1] 21817
```

```
mycounts <- meancounts[to.keep,]
head(mycounts)
```

```
                control.mean treated.mean      log2fc
ENSG00000000003       900.75       658.00 -0.45303916
ENSG00000000419       520.50       546.00  0.06900279
ENSG00000000457       339.75       316.50 -0.10226805
ENSG00000000460        97.25        78.75 -0.30441833
ENSG00000000971      5219.00      6687.50  0.35769358
ENSG00000001036      2327.00      1785.75 -0.38194109
```

Q6. How many genes are "up" regulated at a log2fc threshold of +2?

```
sum(mycounts$log2fc >= 2)
```

```
[1] 314
```

Q7. How many genes are "down" regulated at a log2fc threshold of -2?

```
sum(mycounts$log2fc <= -2)
```

```
[1] 485
```

Hold on... we're missing stats.

DESeq2 Analysis

Let's do this properly and consider the stats - are the differences in the means significant?

We will use DESeq2 to do this:

```
library(DESeq2)
```

The first function we will use from this package sets up the input in the particular format that DESeq wants:

```
dds <- DESeqDataSetFromMatrix(countData = counts,
                        colData = metadata,
                        design = ~dex)
```

```
converting counts to integer mode
```

```
Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
design formula are characters, converting to factors
```

We can now run our DESeq analysis

```
dds <- DESeq(dds)
```

```
estimating size factors
```

```
estimating dispersions

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

```
res <- results(dds)
```

Peak at results

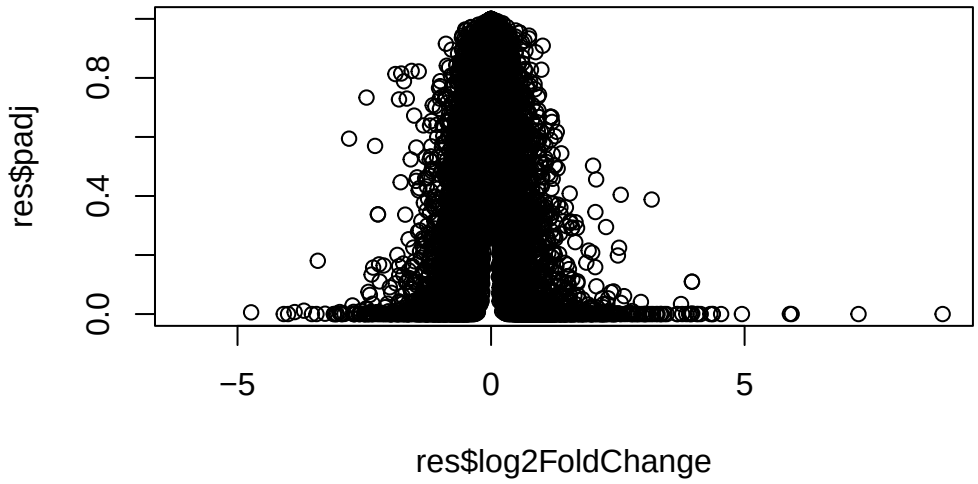
```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
ENSG00000000003  0.163035
ENSG00000000005        NA
ENSG00000000419  0.176032
ENSG00000000457  0.961694
ENSG00000000460  0.815849
ENSG00000000938        NA
```

Result figure: Volcano Plots

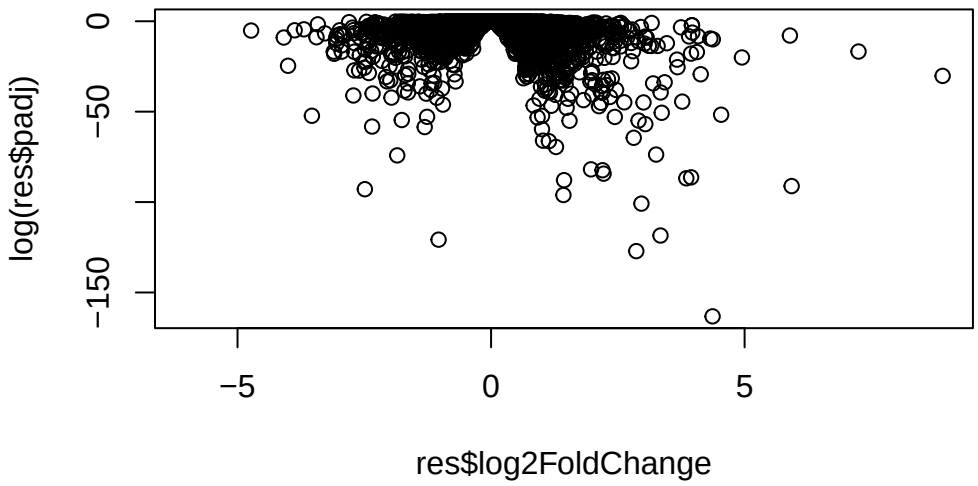
Plot of Log2FC vs. P-value

```
plot(res$log2FoldChange, res$padj)
```

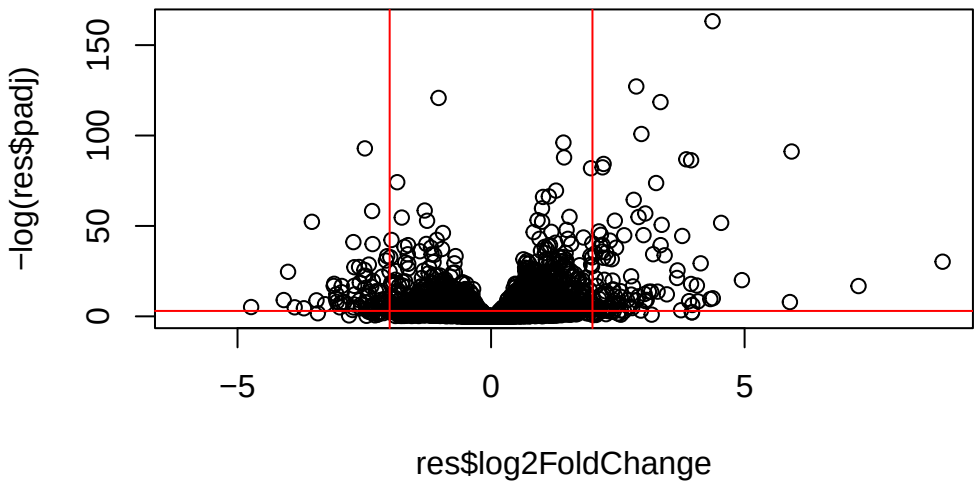


This P-value data is again heavily skewed so lets log transform

```
plot(res$log2FoldChange, log(res$padj))
```




```
plot(res$log2FoldChange, -log(res$padj))
abline(v=-2, col="red")
abline(v=+2, col="red")
abline(h=-log(0.05), col="red")
```



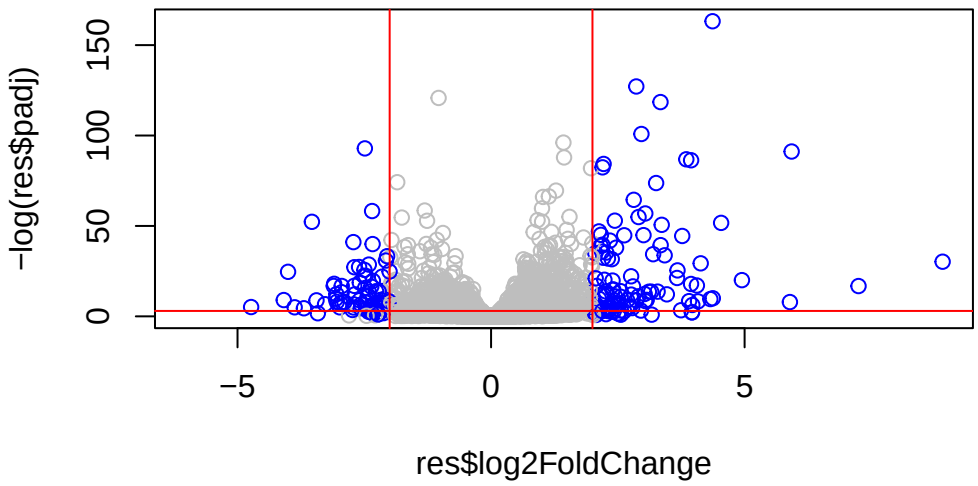
Let's add some color.

```
mycols <- rep("gray", nrow(res))
mycols[ res$log2FoldChange <= -2 ] <- "blue"
mycols[ res$log2FoldChange >= 2 ] <- "blue"
mycols[ res$padj >=0.5 ] <- "gray"

plot(res$log2FoldChange, -log(res$padj), col=mycols) +
abline(v=-2, col="red")
```

```
integer(0)
```

```
abline(v=+2, col="red")
abline(h=-log(0.05), col="red")
```




```
#mycols
```

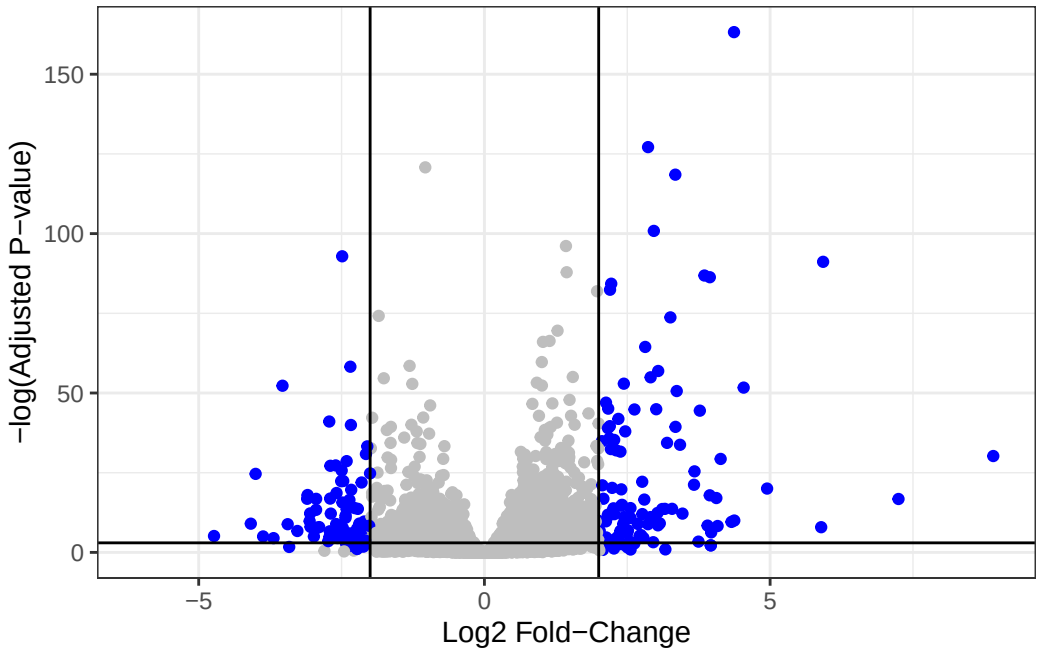
```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
ENSG00000000003  0.163035
ENSG00000000005        NA
ENSG00000000419  0.176032
ENSG00000000457  0.961694
ENSG00000000460  0.815849
ENSG00000000938        NA
```

Q8. Make a ggplot volcano plot with colors and lines as annotation along with nice axis labels.

```
ggplot(as.data.frame(res)) +
  aes(res$log2FoldChange, -log(res$padj)) +
        geom_point(col=mycols) +
  geom_vline(xintercept = c(-2, +2)) +
  geom_hline(yintercept = -log(0.05)) +
  theme_bw() +
  labs(x="Log2 Fold-Change", y="-log(Adjusted P-value)")
```

```
Warning: Removed 23549 rows containing missing values or values outside the scale range
(`geom_point()`).
```



We first need to add gene symbols (e.g. HBB) so we know what genes we're dealing with. We need to "translate" between ENSEMBLE ids that we have in the rownames of `res`.

```
head ( rownames(res) )
```

```
[1] "ENSG00000000003" "ENSG00000000005" "ENSG00000000419" "ENSG00000000457"
[5] "ENSG00000000460" "ENSG00000000938"
```

```
library(AnnotationDbi)
library(org.Hs.eg.db)
```

Different database ID types I can translate between.

```
columns(org.Hs.eg.db)
```

```
 [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
 [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
[11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MAP"
[16] "OMIM"         "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"
[21] "PMID"         "PROSITE"      "REFSEQ"       "SYMBOL"       "UCSCKG"
[26] "UNIPROT"
```

Lets "map" between "ENSEMBL" and "SYMBOL" (i.e. gene symbol).

```
res$symbol <- mapIds(x = org.Hs.eg.db,
       keys = rownames(res),
       keytype = "ENSEMBL",
       column = "SYMBOL")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 7 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj      symbol
```

```
                <numeric> <character>
ENSG00000000003  0.163035      TSPAN6
ENSG00000000005        NA        TNMD
ENSG00000000419  0.176032        DPM1
ENSG00000000457  0.961694       SCYL3
ENSG00000000460  0.815849       FIRRM
ENSG00000000938        NA         FGR
```

Add a few more ID mappings including "GENENAME" and "ENTREZID".

```
res$name <- mapIds(x = org.Hs.eg.db,
       keys = rownames(res),
       keytype = "ENSEMBL",
       column = "GENENAME")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$entrez <- mapIds(x = org.Hs.eg.db,
       keys = rownames(res),
       keytype = "ENSEMBL",
       column = "ENTREZID")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 9 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
                     padj      symbol                   name      entrez
                <numeric> <character>            <character> <character>
ENSG00000000003  0.163035      TSPAN6          tetraspanin 6        7105
```

```
ENSG00000000005        NA        TNMD            tenomodulin       64102
ENSG00000000419  0.176032        DPM1 dolichyl-phosphate m..        8813
ENSG00000000457  0.961694       SCYL3 SCY1 like pseudokina..       57147
ENSG00000000460  0.815849       FIRRM FIGNL1 interacting r..       55732
ENSG00000000938        NA         FGR FGR proto-oncogene, ..        2268
```

Be sure to save our annotated results to a file.

```
write.csv(res, file="my_annotated_results.csv")
```

Pathway analysis

Install the packages we need for pathway analysis: run in your R console (i.e. not your Quarto doc!) `BiocManager::install( c("pathview", "gage", "gageData") )`

```
library(pathview)
library(gage)
library(gageData)
```

Let's peak at the gageData

```
data(kegg.sets.hs)

# Examine the first 2 pathways in this kegg set for humans
head(kegg.sets.hs, 2)
```

```
$`hsa00232 Caffeine metabolism`
[1] "10"   "1544" "1548" "1549" "1553" "7498" "9"

$`hsa00983 Drug metabolism - other enzymes`
 [1] "10"     "1066"   "10720"  "10941"  "151531" "1548"   "1549"   "1551"
 [9] "1553"   "1576"   "1577"   "1806"   "1807"   "1890"   "221223" "2990"
[17] "3251"   "3614"   "3615"   "3704"   "51733"  "54490"  "54575"  "54576"
[25] "54577"  "54578"  "54579"  "54600"  "54657"  "54658"  "54659"  "54963"
[33] "574537" "64816"  "7083"   "7084"   "7172"   "7363"   "7364"   "7365"
[41] "7366"   "7367"   "7371"   "7372"   "7378"   "7498"   "79799"  "83549"
[49] "8824"   "8833"   "9"      "978"
```

To run pathway analysis we will use the `gage()` function and it requires a wee "vector of importance". We will use our Log2FC results from our `res` object.

```
foldchanges = res$log2FoldChange
names(foldchanges) = res$entrez
head(foldchanges)
```

```
       7105       64102        8813       57147       55732        2268
-0.35070302          NA  0.20610777  0.02452695 -0.14714205 -1.73228897
```

```
# Get the results
keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

What's in the returned `keggres` object

```
attributes(keggres)
```

```
$names
[1] "greater" "less"    "stats"
```

```
head(keggres$less)
```

```
                                                      p.geomean stat.mean
hsa05332 Graft-versus-host disease                   0.0004250461 -3.473346
hsa04940 Type I diabetes mellitus                    0.0017820293 -3.002352
hsa05310 Asthma                                      0.0020045888 -3.009050
hsa04672 Intestinal immune network for IgA production 0.0060434515 -2.560547
hsa05330 Allograft rejection                         0.0073678825 -2.501419
hsa04340 Hedgehog signaling pathway                  0.0133239547 -2.248547
                                                            p.val      q.val
hsa05332 Graft-versus-host disease                   0.0004250461 0.09053483
hsa04940 Type I diabetes mellitus                    0.0017820293 0.14232581
hsa05310 Asthma                                      0.0020045888 0.14232581
hsa04672 Intestinal immune network for IgA production 0.0060434515 0.31387180
hsa05330 Allograft rejection                         0.0073678825 0.31387180
hsa04340 Hedgehog signaling pathway                  0.0133239547 0.47300039
                                                     set.size         exp1
hsa05332 Graft-versus-host disease                         40 0.0004250461
hsa04940 Type I diabetes mellitus                          42 0.0017820293
hsa05310 Asthma                                            29 0.0020045888
```

```
hsa04672 Intestinal immune network for IgA production       47 0.0060434515
hsa05330 Allograft rejection                                36 0.0073678825
hsa04340 Hedgehog signaling pathway                         56 0.0133239547
```

We can pass our foldchange vector (our results) together with any of these highlighted pathway IDs to see how our genes

```
pathview(gene.data=foldchanges, pathway.id="hsa05310")
```

```
'select()' returned 1:1 mapping between keys and columns
```

```
Info: Working in directory /Users/sarahmirsaidi/Desktop/BIMM 143/class13
```

```
Info: Writing image file hsa05310.pathview.png
```

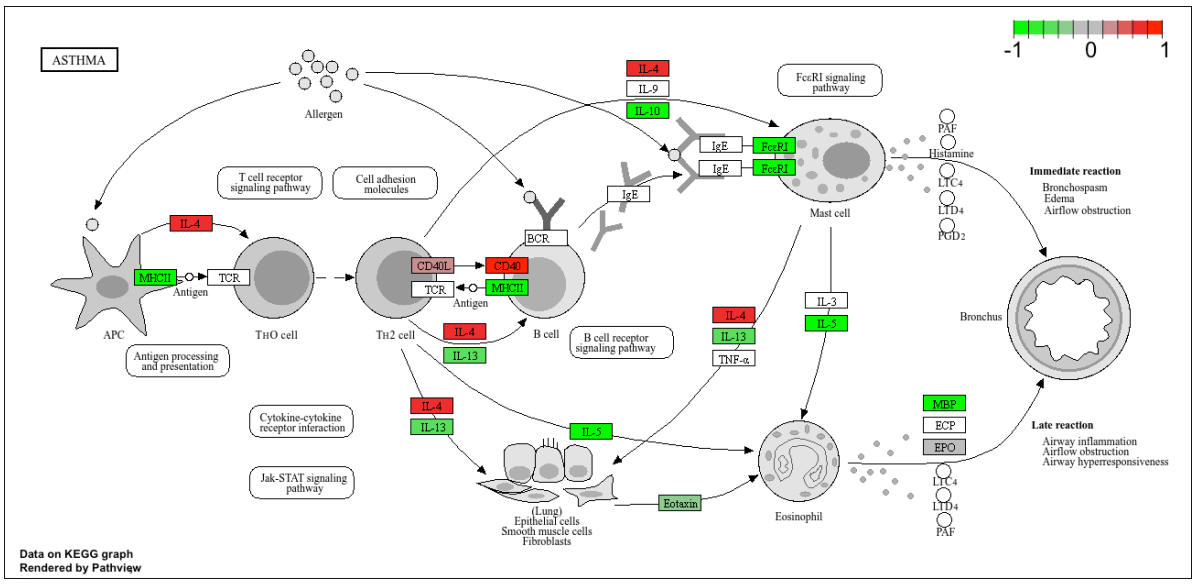


Figure 1: The Asthma pathway overlaps with our differentially expressed genes